(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2001 (01.11.2001)

PCT

(10) International Publication Number
WO 01/81533 A1

(51) International Patent Classification[7]: C12M 3/00

(21) International Application Number: PCT/IT01/00196

(22) International Filing Date: 20 April 2001 (20.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
TO00A000389 21 April 2000 (21.04.2000) IT

(71) Applicant *(for all designated States except US)*: **IGEA S.R.L.** [IT/IT]; Via Parmenide, 10/A, I-41012 Carpi (IT).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MIKLAVCIC, Damijan** [SI/SI]; University of Ljubljana, Faculty of Electrical Engineering, Trzaska 25, 1000 Ljubljana (SI). **MIR, Lluis** [FR/FR]; c/o Institut Gustave-Roussy, UMR 8532 CNRS, 39, rue Camille Desmoulins, F-94805 Villejuif Cedex (FR).

(74) Agents: **JORIO, Paolo** et al.; c/o Studio Torta S.r.l., Via Viotti, 9, I-10121 Torino (IT).

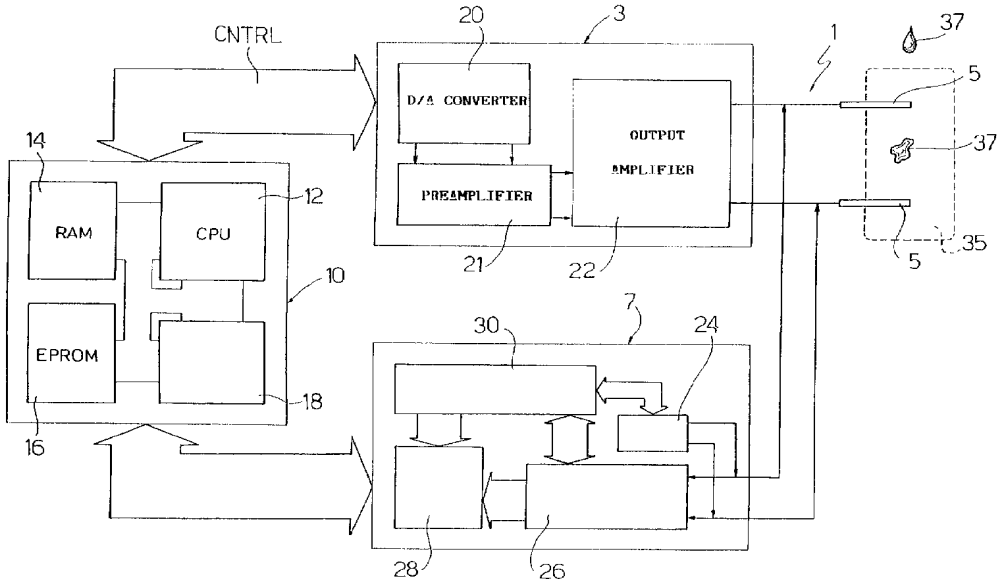
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

[Continued on next page]

(54) **Title:** ELECTROPORATION DEVICE AND METHOD, WHERE AMPLITUDE OF THE ELECTRIC PULSE OR PULSES IS AUTOMATICALLY SET ACCORDING TO PRE-PULSE MEASUREMENT OF ELECTRIC PROPERTIES OF THE SAMPLE



(57) **Abstract:** A method for electroporation of a substrate (35) containing cells, the method including the steps of: determining (7, 10, 3, 100) the impedance of the substrate (35); calculating an objective value (Vo) of a signal on the basis of the predetermined impedance; and supplying the substrate (35), by means of electrodes (5), with a voltage, the value of which is correlated to the previously calculated objective value (Vo).

WO 01/81533 A1

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ELECTROPORATION DEVICE AND METHOD, WHERE AMPLITUDE OF THE ELECTRIC PULSE OR PULSES IS AUTOMATICALLY SET ACCORDING TO PRE-PULSE MEASUREMENT OF ELECTRIC PROPERTIES OF THE SAMPLE

TECHNICAL FIELD

The present invention relates to an electroporation device and method, where amplitude of the electric pulse or pulses is automatically set according to pre-pulse measurement of electric properties of the sample.

BACKGROUND ART

As is known, recent biological, microbiological and pharmacological applications involve introducing molecules into cells, which is done by inserting the molecules through the cell membranes.

The molecules may be inorganic substances (e.g. drugs) or organic molecules (cells are known to be inserted, for example, with DNA molecules).

Molecules are introduced using various methods, including:

viral vectoring : associating the molecule with a virus, which is then introduced into the cell;

chemical vectoring : associating the molecule with a

chemical substance for reducing the resistance of the cell membrane and so permitting introduction of the molecule into the cell; and

ballistic methods : accelerating the molecule so that it strikes and penetrates the cell membrane.

Known methods involve several drawbacks, including: risk of immunity reaction to the vector; production difficulties and poor stability of the vector itself (viral vectoring); ineffectiveness, toxicity and poor selectivity (chemical vectoring). As for ballistic methods, these only apply to surface cells.

New so-called electroporation methods have recently been devised, which provide for briefly applying a strong electric field to the cells to permeabilize, and so enable substances to penetrate, the cell membrane.

One problem posed by known electroporation methods is establishing the value of the electric field applied, which must be high enough to permeabilize the cell membrane, but not so high as to cause irreversible damage to the cell.

More specifically, known electroporation devices and methods employ a fixed output voltage (determined, for example, experimentally), which in certain operating conditions, may be too low, thus preventing introduction of the substances, or too high, thus resulting in irreversible damage to the cell.

DISCLOSURE OF INVENTION

It is an object of the present invention to provide

an electroporation device and method designed to eliminate the drawbacks of known electroporation devices and methods.

According to the present invention, there is provided an electroporation device as described in Claim 1.

The present invention also relates to an electroporation method as described in Claim 7.

BRIEF DESCRIPTION OF THE DRAWINGS

A preferred, non-limiting embodiment of the invention will be described by way of example with reference to the accompanying drawings, in which:

Figure 1 shows, schematically, an electroporation device in accordance with the teachings of the present invention;

Figure 2 shows a logic operating diagram of the Figure 1 device; and

Figure 3 shows signals produced by the Figure 1 device.

BEST MODE FOR CARRYING OUT THE INVENTION

Number 1 in Figure 1 indicates as a whole an electroporation device.

Device 1 comprises a signal generator, in particular a voltage pulse generator 3 having at least two output electrodes 5; a measuring system 7 connected to output electrodes 5; and an electronic control unit 10 for controlling voltage pulse generator 3 and measuring system 7.

Electronic control unit 10 comprises at least one microprocessor 12 cooperating with memory devices, e.g. a RAM memory 14 and EPROM memory 16; and interface devices 18.

Pulse generator 3 comprises a digital-analog D/A converter 20, which receives a control signal CNTRL from unit 10 and cooperates at the output with a preamplifying circuit 21; preamplifying circuit 21 has an output connected to the input of a power amplifier 22 in turn having an output communicating with electrodes 5; and electrodes 5, in the example embodiment shown, are each defined by a flat, rectangular metal blade to which the output signal from power amplifier 22 is applied.

The electrodes may, of course, differ in shape, structure and size from those shown, e.g. may be designed for use in a laparoscopy process.

Measuring system 7 comprises an oscillating circuit 24 for supplying electrodes 5 with an excitation signal; and a converting circuit 26 supplied by electrodes 5 with a signal in response to the excitation signal. Converting circuit 26 cooperates with a memory 28 (e.g. a RAM memory) which is also connected to a known measuring circuit 30, which also cooperates with converting circuit 26 and with oscillating circuit 24. The measure of impedance may be done in the frequency domain or in the time domain.

Figure 2 shows a block diagram of the operations performed by electroporation device 1 under the control

of electronic unit 10.

When device 1 is activated, a first block 100 determines the impedance value between electrodes 5. More specifically, the impedance $Z(\omega)$ is measured in known manner by measuring system 7, which may determine one or several of the following parameters for instance the absolute impedance value $|Z(\omega)|$, the real impedance part Zr, the imaginary part jZo, or angle $\alpha = \text{arctg}(Zo/Zr)$.

Alternatively or in addition to the measure of impedance above described the device may also measure other electric characteristics such as admittance, resistivity or conductivity including dynamic resistance or dynamic conductivity. The device may also measure current at constant voltage and vice versa.

If the measured impedance $Z(\omega)$ is below a threshold value $Zsgl(\omega)$, i.e. $Z(\omega) < Zsgl(\omega)$ (e.g. the absolute impedance value is less than the threshold value), operation is arrested automatically with no pulse being emitted (block 110 downstream from block 100).

If the measured impedance $Z(\omega)$ is above (or equal to) the threshold value $Zsgl(\omega)$, i.e. $Z(\omega) => Zsgl(\omega)$ (e.g. the absolute impedance value is greater than or equal to the threshold value), block 110 is followed by a block 120, which calculates an objective voltage value Vo on the basis of the measured impedance value $Z(\omega)$.

The control by block 110 is normally performed when the device 1 uses a particular multiple electrode (not shown) comprising a number of couples of electrodes 5. In this

case, the signal outputted by pulse generator 5 is sequentially supplied, by means of a multiplexer device (not shown), to the couples of electrodes applied to a substrate containing cells to realize the permeabilization of the substrate. In this case, the substrate connected between electrode 5 whose impedance is measured by block 100 may be already permeabilized by the signal produced by another couple of electrode. If no multiple electrode is used and therefore no initial permeabilization caused by a signal outputted by other electrodes is present block 110 may be omitted.

Objective voltage Vo may be calculated by means of a table (not shown) in which input impedance values are associated with output objective voltage values. In an alternative embodiment, the objective voltage may be calculated by means of a mathematical function (e.g. a linear function not shown), which supplies an output objective voltage value Vo on the basis of input impedance values.

More specifically, the table or function provides for a direct proportional application wherein the objective voltage value Vo is low as impedance is low, and is high as impedance is high.

Block 120 is followed by a block 130, which generates a first control signal CNTRL1 for pulse generator 3, which, in response, produces a first number of first voltage pulses I1 which are applied to electrodes 5 (Figure 3).

First pulses I1, which are preferably rectangular, have an amplitude corresponding with the objective voltage value Vo calculated previously.

In a preferred embodiment of the invention, a predetermined number of first pulses I1 are produced, so as to control the amplitude of pulses I1.

After block 130 the process may stop.

In an alternative embodiment block 130 may be followed by block 140, which generates a further control signal CNTRLu for pulse generator 3, which, in response, produces further voltage pulses If which are applied to electrodes 5 (Figure 3).

Further pulses If may comprise:

. rectangular first further pulses If1, the amplitude of which is closed-loop adjustable on the basis of the instantaneous measured impedance value; more specifically, the amplitude of first further pulses If1 decreases as impedance falls, and increases as impedance rises or remains constant;

. rectangular second further pulses If2 of fixed amplitude; more specifically, pulses If2 have a lower amplitude and a greater time width than first pulses I1; and

. a series of pulses comprising first further pulses If1 and second further pulses If2.

Application of further pulses If is regulated by a block 150 downstream from block 140 and which arrests generation of further pulses If when at least one

operating condition is met. The following are possible operating conditions indicated purely by way of non-limiting examples:

. a first operating condition wherein a predetermined number of further pulses If have been generated;

. a second operating condition wherein further pulses If have been generated for a predetermined time; and

. a third operating condition wherein a given instantaneous impedance $Z(\omega)$ measured between electrodes 5 has been reached.

In actual use, electrodes 5 are applied to a tissue portion 35 (shown schematically in Figure 1) containing live cells. The tissue portion may be one forming part of a live being (human, animal or vegetable) or one containing cells removed from a live being (human, animal or vegetable). Tissue portions are also understood to include cultures of uni- or multicellular organisms. In other words, a tissue portion is intended to mean, in general, a substrate of any nature on which live cells or cellular organisms are present.

Tissue portion 35 is also applied with a substance (organic or inorganic) 37 to be introduced into the cells. The substance may be applied in a number of different ways, some of which are listed below by way of non-limiting examples:

. direct application of the substance to the tissue portion, e.g. by applying the tissue portion with a fluid containing the substance;

. indirect application of the substance, e.g. by introducing the substance into the circulatory system of the tissue portion;

. injecting the substance, e.g. using needlelike electrodes 5, each having an inner conduit containing the substance to be injected into the tissue portion. Needles separate from electrodes 5 may, of course, also be used.

The substance introduced may be inorganic or organic, e.g.

. a DNA molecule containing one or more regulatory sequences and/or sequences coding for therapeutic genes or genes of interest for biomedical or biotechnological purposes;

. an oligonucleotide, whether natural (phosphodiesters) or modified (inside the backbone of the oligonucleotide, such as phosphosulfates, or at the extremities, by addition of groups to protect the oligonucleotides from digestion by nucleases) - the description of oligonucleotide modification being non-limiting;

. a protein or peptide, whether natural or genetically or chemically modified, obtained by natural means or by synthesis, or a molecule mimicking the structure of a protein or peptide, whatever its chemical backbone;

. a cytotoxic agent; in particular, of cytotoxic agents, the antibiotic bleomycin or cisplatinum;

. a penicillin;

. a nucleic acid;

. a pharmacological agent other than a nucleic acid.

Device 1 is activated to immediately determine the impedance of tissue 35 whose value depends on the electrode geometry and tissue type and the permeability of the cell membranes. If the impedance is too low (block 110), i.e. if the cell membranes are already permeabilized for instance because a multiple electrode has been used and the signal outputted by other electrode has already permeabilized tissue 35, no voltage pulse is generated to prevent damaging tissue 35.

Conversely, if the measured impedance value is acceptable - in particular, above the threshold value (block 110) - voltage pulses I1 are generated, the value of which depends on the previously measured impedance. That is, a low-amplitude pulse is generated if the tissue is already partly permeabilized, in the case of use of multiple electrode, and a higher-amplitude pulse if the tissue is permeabilized poorly. The intensity of the electric field applied is thus related to the actual electric characteristics of the tissue, which in turn depend on the extent to which the cell membranes are permeabilized, thus preventing application of a too high electric field and cell damage in the event the tissue is already permeabilized.

First pulses I1 are thus generated and applied to electrodes 5 to produce an electric field which is directed into the tissue portion to initiate permeabilization of the tissue cell membranes.

Following emission of a predetermined number of

first pulses (block 130), further pulses If may be applied to ensure complete permeabilization of tissue 35. In particular, pulses If may promote the introduction of high-molecular-weight substances, e.g. DNA.

Substance 37 is then introduced into the cells.

The knowledge gathered by the Applicant indicates that applying at least one pulse I1 of an amplitude adjustable according to the impedance measurement provides for achieving a high degree of permeabilization of the cell membranes, while at the same time preventing damage to the cell tissue.

Clearly, changes may be made to the device as described herein without, however, departing from the scope of the present invention.

As opposed to being measured using the same pair of electrodes 5 used to apply the electric field to substrate 35, as in the embodiment described above, the impedance of substrate 35 may also, obviously, be measured by a separate pair of auxiliary electrodes (not shown) close to electrodes 5 and placed in contact with tissue portion 35 to be permeabilized.

CLAIMS

1) An electroporation device comprising signal generating means (3) connectable at the output to electrodes (5) fittable to a substrate (35) comprising cells; said electrodes (5) producing, in said substrate (35), an electric field which induces permeabilization of the membranes of said cells to facilitate introduction of substances (37) into the cells; characterized by comprising:

- measuring means (7, 10, 3, 100) for determining at least one electric characteristic (Z(ω)) of said substrate (35); said electric characteristic (Z(ω)) being a function of the permeabilization of the membranes of said cells;

- calculating means (120) for receiving at the input the determined said electric characteristic (Z(ω)) and generating at the output an objective value (Vo) of said signal; and

- application means (130) for supplying said electrodes with an electric signal (I1), the value of which is correlated to the previously calculated said objective value (Vo).

2) A device as claimed in Claim 1, wherein said measuring means (7, 10, 3, 100) determine the impedance (Z(ω)) of the substrate (35); said calculating means generating said objective value as a function of the measured impedance.

3) A device as claimed in Claim 1 or 2, wherein said application means (130) apply to said substrate at least one voltage pulse (3), the amplitude of which is correlated to said objective value (Vo).

4) A device as claimed in Claim 1 or 2, wherein said application means (130) apply (3) to said substrate a number of voltage pulses (I1); the amplitude of each pulse (I1) being correlated to said objective value (Vo).

5) A device as claimed in any one of the foregoing Claims wherein further signal generating means (140) are provided, which are activated after said application means (130) and generate at least a first further pulse (If1) having an amplitude which is closed-loop adjustable on the basis of the instantaneous value of the impedance measured in said substrate (35); the amplitude of said first further pulses (If1) decreasing alongside a reduction in impedance, and increasing as impedance increases or remains noticeably constant.

6) A device as claimed in any one of the foregoing Claims, wherein further signal generating means (140) are provided, which are activated after said application means (130) and generate second further pulses (If2) of a given, in particular, rectangular shape and of fixed amplitude.

7) A method for electroporation of a substrate (35) containing cells, for introducing at least one substance (37) into the cells; characterized by comprising the steps of:

- determining (7, 10, 3, 100) at least one electric characteristic (Z(ω)) of said substrate (35); said electric characteristic (Z(ω)) being a function of the permeabilization of the membranes of said cells;

- calculating (120) an objective value (Vo) of a signal on the basis of the determined said electric characteristic (Z(ω)); and

- supplying (130, 5) said substrate (35) with an electric signal, the value of which is correlated to the previously calculated said objective value (Vo).

8) A method as claimed in Claim 7, wherein said determining step (7, 10, 3, 100) comprises the step of measuring the impedance (Z(ω)) of the substrate (35).

9) A method as claimed in Claim 7 or 8, wherein said step (130, 5) of supplying an electric signal comprises the step of generating (3) at least one voltage pulse, the amplitude of which is correlated to the previously calculated said objective value (Vo).

10) A method as claimed in Claim 7, characterized in that said substance is selected from a list comprising:

- a nucleic acid;

. a DNA molecule;

. an oligonucleotide;

- a protein;

- a peptide;

. a cytotoxic agent, in particular the antibiotic bleomycin or cisplatinum;

. a penicillin;

- a pharmacological agent other than a nucleic acid.

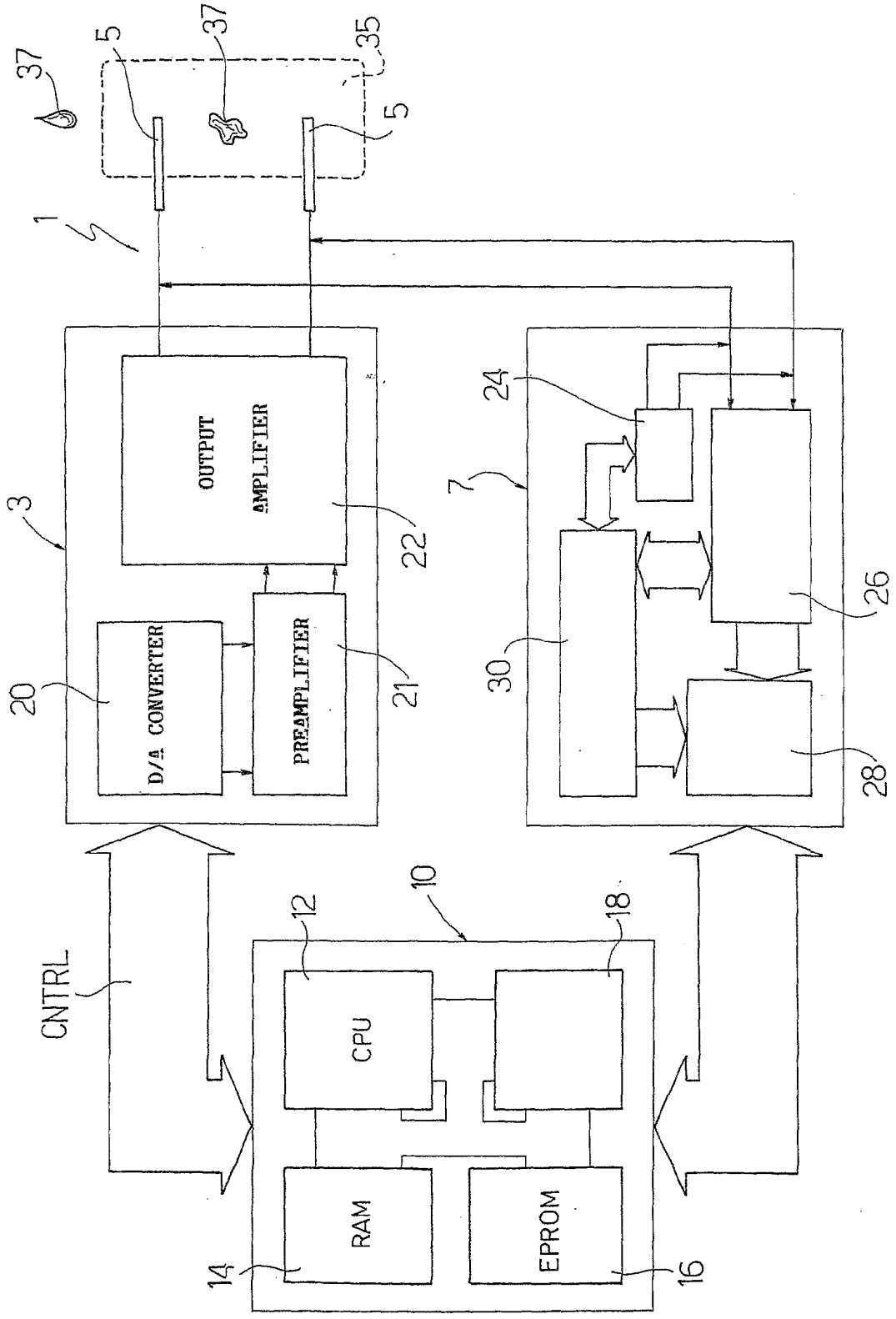

37
5
37
35
5
1
OUTPUT AMPLIFIER
22
3
D/A CONVERTER
20
PREAMPLIFIER
21
24
7
26
30
28
CNTRL
10
12
18
CPU
RAM
14
EPROM
16

Fig. 1

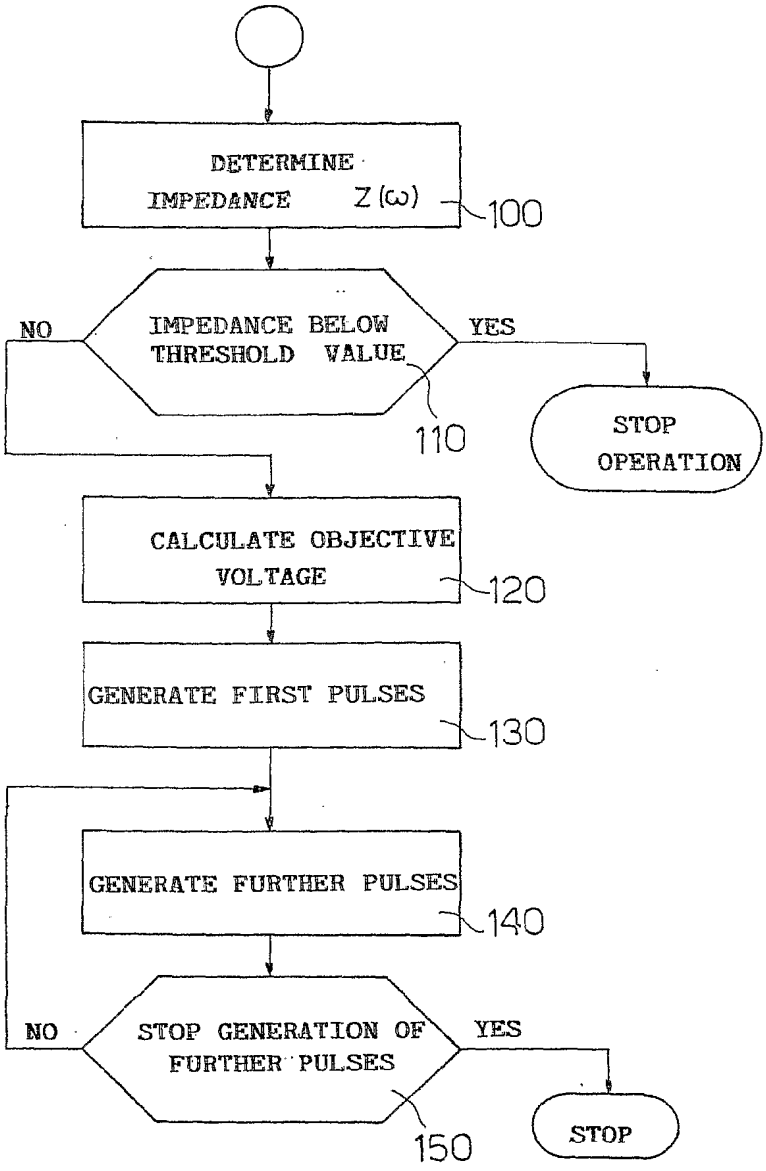

DETERMINE IMPEDANCE Z(ω)
100
IMPEDANCE BELOW THRESHOLD VALUE
NO
YES
110
STOP OPERATION
CALCULATE OBJECTIVE VOLTAGE
120
GENERATE FIRST PULSES
130
GENERATE FURTHER PULSES
140
STOP GENERATION OF FURTHER PULSES
NO
YES
150
STOP

Fig. 2

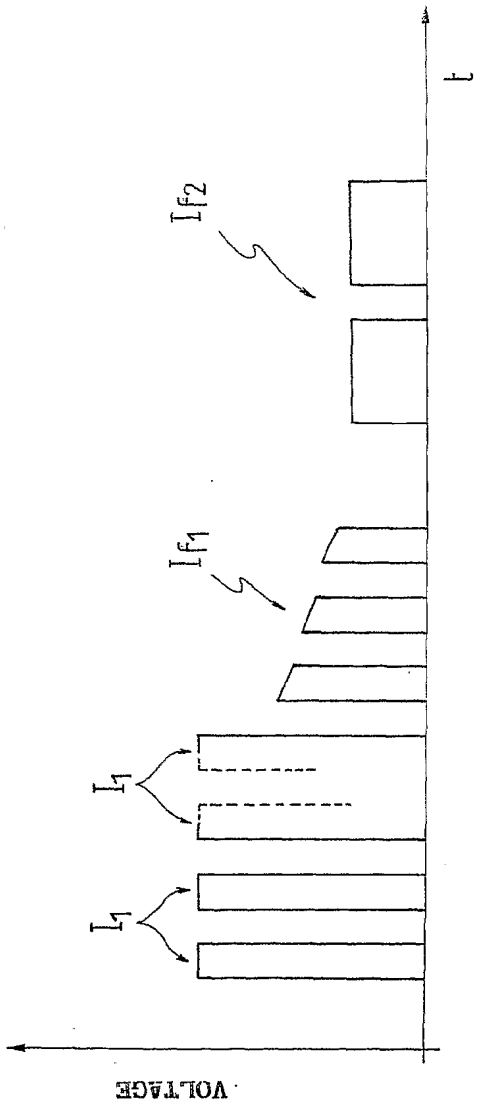


Fig.3

INTERNATIONAL SEARCH REPORT

Intel onal Application No
PCT/IT 01/00196

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12M3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, IBM-TDB

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 01 07583 A (UNIV CALIFORNIA) 1 February 2001 (2001-02-01) page 9, paragraph 2 - paragraph 3; claims 1,2,11,17	1,2,7,8, 10
P,X	WO 01 07584 A (UNIV CALIFORNIA) 1 February 2001 (2001-02-01) claims 1,2,11,17	1,2,7,8, 10
X	US 5 134 070 A (CASNIG DAEL R) 28 July 1992 (1992-07-28) column 16, line 15 - line 26	1,10
A	WO 00 20554 A (SHAW JOHN EDWARD ANDREW ;BRENNAN DAVID (GB); DODGSON JOHN (GB); ZE) 13 April 2000 (2000-04-13) page 11, paragraph 2	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 2001	05/10/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Coucke, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IT 01/00196

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 39531 A (MASSACHUSETTS INST TECHNOLOGY) 12 December 1996 (1996-12-12) claims	1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ıal Application No
PCT/IT 01/00196

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0107583	A	01-02-2001	AU	6231400 A	13-02-2001
			AU	6364200 A	13-02-2001
			AU	6365000 A	13-02-2001
			WO	0107583 A1	01-02-2001
			WO	0107584 A1	01-02-2001
			WO	0107585 A1	01-02-2001
WO 0107584	A	01-02-2001	AU	6231400 A	13-02-2001
			AU	6364200 A	13-02-2001
			AU	6365000 A	13-02-2001
			WO	0107583 A1	01-02-2001
			WO	0107584 A1	01-02-2001
			WO	0107585 A1	01-02-2001
US 5134070	A	28-07-1992	CA	2025022 A1	08-09-1991
WO 0020554	A	13-04-2000	AU	6216199 A	26-04-2000
			EP	1124939 A1	22-08-2001
			WO	0020554 A1	13-04-2000
WO 9639531	A	12-12-1996	US	5749847 A	12-05-1998
			WO	9639531 A1	12-12-1996